

Role of oxidative stress mediated by glutathione-S-transferase in thiopurines toxic effects

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Abstract

Azathioprine (AZA), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are antimetabolite drugs, widely used as immunosuppressants and anticancer agents. Despite their proven efficacy, high incidence of toxic effects in patients during the standard-dose therapy is recorded. The aim of this study is to explain, from a mechanistic point of view, the clinical evidence showing a significant role of glutathione-S-transferase (GST)-M1 genotype on AZA toxicity in inflammatory bowel disease patients.

To this aim, the human non-tumor IHH and HCEC cell lines were chosen as predictive models of the hepatic and intestinal tissues, respectively. AZA, but not 6-MP and 6-TG, induced a concentration-dependent superoxide anion production that seemed dependent on GSH depletion. N-acetyl-cysteine reduced AZA anti-proliferative effect in both cell lines and GST-M1 overexpression increased both superoxide anion production and cytotoxicity, especially in transfected HCEC cells.

In this study, an *in vitro* model to study thiopurines metabolism has been set up and helped us to demonstrate, for the first time, a clear role of GST-M1 in modulating AZA cytotoxicity, with a close dependency on superoxide anion production. These results provide the molecular basis to shed light on the clinical evidence suggesting a role of GST-M1 genotype in influencing the toxic effects of AZA treatment.

1 Introduction

Thiopurines, azathioprine (AZA), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), are antimetabolite drugs, thio-analogues of purine bases, widely used as effective immunosuppressants; 6-MP and 6-TG are also employed as antileukemic drugs.¹ AZA is the pro-drug of 6-MP, commonly used as an immunosuppressant for the treatment of various chronic inflammatory diseases, such as inflammatory bowel disease (IBD), and following organ transplantation.^{2, 3} Thiopurine antimetabolites are inactive pro-drugs and, in order to exert their cytotoxic action, must be converted into the active metabolites 6-thioguanine nucleotides (6-TGNs), which are substrates for incorporation into DNA.⁴ Despite the proven efficacy of these drugs, adverse reactions to thiopurines occur in a high percentage of patients, during the standard-dose therapy, while some patients do not respond to therapy at all.⁵ The inter-individual variability in the response to thiopurines is in part linked to the complexity of their intracellular metabolism that is mediated by several enzymes. The presence of polymorphisms in genes encoding for these enzymes, that induces alterations in their activity, can be responsible of this variability. One of the most studied examples is that of thiopurine-S-methyltransferase (TPMT) polymorphisms.⁶ Given the enzymes involved in thiopurines metabolism, oxidative stress could have a role in their effects at the cellular level; this seems likely especially for AZA, that is activated to 6-MP through a conjugation reaction with reduced glutathione (GSH), which can lead to the depletion of the cytosolic pool of GSH.⁷ This conversion can occur spontaneously¹ but *in vitro* studies have indicated a relevant role of the enzymes glutathione-S-transferase (GST)⁸ and in particular of the isoforms GST-A1/2 and GST-M1.⁹ Moreover, in recent studies conducted by our group in young subjects with IBD, we found that patients with reduced levels of GST-M1, due to genetic deletion, present decreased sensitivity to the effects of AZA¹⁰ and a decreased amount of active 6-TGNs,¹¹ putatively because of a reduced GST-catalyzed biotransformation of AZA to 6-MP.¹² GSTs

may contribute to AZA effects not only by increasing the activation of AZA to 6-MP but also modulating GSH consumption and oxidative stress which is more evident in cells with high GST activity compared with those with low or absent activity, leading to higher cellular and tissue damage.^{13, 14} Because GSH is the main antioxidant system in the cell, its depletion induces significant reactive oxygen species (ROS) accumulation, as demonstrated in several systems.^{15, 16} Production of ROS during treatment with thiopurines could be related also to xanthine oxidase (XO), a known ROS producing enzyme which, in the liver, plays an important role in the first-pass metabolism of these drugs after oral administration, catabolizing 6-MP to the inactive thiouric acid. Indeed, in primary cultures of rat hepatocytes incubated with thiopurines, metabolism of these drugs by XO may generate ROS.^{17, 18} Allopurinol is a powerful XO inhibitor which improves response to thiopurines by increasing the concentration of active 6-TGNs.¹⁹

Drug activation and some severe adverse effects of thiopurines occur in the intestinal and hepatic tissues and elucidation of molecular events specific of these tissues may be of interest to improve efficacy and safety of the treatment with these agents.²⁰ To this end, we investigated *in vitro* the sensitivity to thiopurines in stabilized non tumor human cell lines of hepatic and intestinal origin evaluating the contribution of oxidative stress in the mechanism of action of these drugs.

2 Experimental procedures

2.1 Materials

Thiopurines and all reagents for cell culture were purchased from Sigma-Aldrich (Milan, Italy). The empty pCMV6 plasmids (PS100001), the pCMV6 plasmids carrying the cDNA sequence encoding for GSTM-1 and DDK (RC223332) and anti-DDK antibodies were obtained from Origene (Milan, Italy). Rabbit anti-actin was from Millipore (Milan, Italy), secondary anti-rabbit and anti-mouse HRP-conjugated antibodies from Cell Signaling Technology (Milan, Italy) and the LiteAblot[®] TURBO kit was from Euroclone (Milan, Italy). The X-TremeGENE 9 DNA Transfection Reagent was from Roche (Milan, Italy) and the restriction enzyme AatI from Thermo Scientific (Milan, Italy). All the other reagents of analytic grade were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Cell cultures

The immortalized human hepatic IHH cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose with the addition of 10% fetal bovine serum (FBS), 1.25% L-glutamine 200 mM, 1% penicillin 10000 UI/ml, streptomycin 10 mg/ml, 1% Hepes buffer 1 M, 0.01 % human insulin 10^{-4} M and 0.04% dexamethasone 1 mg/ml. The non-tumor intestinal human colon epithelium HCEC cell line was maintained in DMEM low glucose with the addition of 10% FBS, 1% L-glutamine 200 mM, 1% penicillin 10000 UI/ml, streptomycin 10 mg/ml, 2% Hepes buffer 1 M, 1% sodium pyruvate 100 mM, 1.5% bovine serum albumin 200 mg/ml, 0.03% ethanolamine 0.166 M, 0.7% O-phosphatidyl-ethanolamine 7×10^{-5} M. Cell cultures were maintained according to standard procedures in humidified incubator at 37°C and with 5% CO₂ and cell passage was performed once a week.

2.3 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay

IHH and HCEC cells (5×10^3 cells/well) were exposed for 96 h to AZA and 6-MP (1.9×10^{-6} – 8.0×10^{-3} M) and 6-TG (3.1×10^{-8} – 1.3×10^{-4} M). Exposure time was chosen on the basis of previously published results.²⁰ In the last 4 h of treatment, a solution of MTT was added (final concentration 0.5 mg/mL) and the crystals solubilized by 100 μ L of DMSO. The absorbance was read by an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, USA) at 540/630 nm. Data are the means \pm SE of at least three independent experiments performed in triplicate and are reported as % of untreated control (absorbance treated/absorbance untreated control*100).

2.4 Nitro blue tetrazolium (NBT) assay

IHH and HCEC cells (1×10^4 cells/well) were exposed for 8 and 24 h to AZA, 6-MP and 6-TG as described above. ROS production, as superoxide anion, was evaluated by NBT reduction assay as previously described.²¹ Briefly, in the last 2 h of treatment, a solution of NBT was added (final concentration 0.5 mg/mL) and the crystals solubilized by 140 μ L of DMSO and 120 μ L of KOH 2M. The absorbance was read by an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, USA) at 630 nm. Data are the means \pm SE of at least three independent experiments performed in triplicate and are reported as % increase with respect to untreated controls [(absorbance treated-absorbance controls)/absorbance controls]*100].

2.5 Cell proliferation

The effect of AZA, 6-MP and 6-TG on proliferation of IHH and HCEC cells was determined by labeling metabolically active cells with [methyl-3H] thymidine. Cells were seeded into a 96-well plate (5×10^4 cells/well) in the presence of AZA, 6-MP and 6-TG at the same concentration range used for MTT assay. After 91 h of incubation, cells were pulsed with [methyl-3H] thymidine (2.5 μ Ci/ml), and the incubation was continued for additional 5 hours.

Cells were then washed with PBS, trypsinized, collected and the radioactivity of the samples was determined by a Liquid Scintillation Analyzer (Wallac 1450 Microbeta liquid scintillation counter, Perkin Elmer, Milan, Italy). Raw count per minute (cpm) data were converted and normalized to percent of maximal proliferation for each experimental condition ($\text{cpm treated/cpm untreated control} \times 100$).

2.6 Glutathione depletion

IHH and HCEC cells (1×10^5 cells/well) were seeded in 6-wells plates and exposed for 96 h to AZA at a concentration equal to the EC_{50} (concentration giving the 50% of the effect) and EC_{95} (concentration giving the 95% of the effect) values obtained by the MTT assay. After treatment, survived cells were collected in ice-cold PBS, lysed, sonicated and protein content measured using a NanoDrop 2000 spectrophotometer (Euroclone, Milan, Italy). In a 96-wells plate, 10 μl of each cell lysate were added to 10 μl of o-phthalaldehyde 1 mg/ml and 180 μl phosphate buffer (K_3PO_4 0.1 M and EDTA 0.005 M, pH 8) and incubated for 15 minutes. Samples were then read by a Fluorocount microplate Fluorometer (Packard, Germany) using a $\lambda_{\text{ex}}=350$ nm and a $\lambda_{\text{em}}=420$ nm. Data obtained by three experiments performed in duplicate were normalized on protein content and expressed as % of GSH with respect to the untreated controls.

2.7 GST-M1 stable transfection

Overexpression of GST-M1 was performed using the X-tremeGENE 9 Transfection Reagent, according to the manufacturer's instructions and using a commercial plasmid pCMV6 carrying the cDNA sequence encoding for GSTM-1 and for the tag DDK and an empty vector plasmid as control (MOCK). In order to increase the efficiency of integration, plasmid vectors were linearized by digestion with the restriction enzyme AatI, using a ratio of 1.5 U of AatI

for 1 μg of plasmid DNA and incubating for 3 h at 37°C. IHH and HCEC cells (5×10^4 cells/well) were seeded in 96-wells plates 24 h before transfection. On 200 μl of each serum-free cell media, 2 μg of linearized plasmid and 6 μl of XtremeGENE were added, yielding a ratio plasmidic DNA:transfection reagent of 1:3. The mix was incubated for 30 min at room temperature and then 10 μl of the mix were added dropwise to each well. Transfected cells were maintained in the incubator at 37°C for 48 h. Cell media were then changed and transfected cells kept in culture with fresh media containing G-418, as selection agent, at final concentrations of 0.25 mg/ml and 1 mg/ml for IHH and HCEC cells, respectively.

2.8 Western blot

Cells (1×10^7) were cultured as reported above, collected, washed in cold PBS and lysed using a lysis buffer composed by Tris-HCl 10 mM pH 7.4, EDTA 100 mM, NaCl 100 mM, SDS 0.1%, Protease inhibitor cocktail 1%. Samples were then run on 10% acrylamide gels in a Tris-Glycine buffer in a PAGErTM Mini-gel Chamber (Lonza, Milan, Italy) and then semi-dry blotted for 2 h with 50 mA current on PVDF membrane. Membranes were blocked for 1 h with 5% not-fat milk in Tween/tris-buffered salt solution (TTBS) and incubated overnight at 4°C with primary antibodies (rabbit anti-actin 1:20000 and mouse anti-DDK 1:1000). Membranes were then washed twice with TTBS and incubated for 1 h at 37°C with secondary anti-rabbit and anti-mouse HRP-conjugated antibodies, at 1:50000 and 1:25000 dilutions, respectively. Chemiluminescence was developed using LiteAblot[®] TURBO kit following manufacturer's instructions and exposed on Kodak Biomax light film.

2.9 Statistical analysis

Results are presented as mean \pm SE from at least three independent experiments and nonlinear regression of concentration-response data was performed for computing EC₅₀ values using

GraphPad Prism version 4.00 (Prism GraphPad, Inc.; San Diego, CA, USA). Data were analyzed by one-way and two-way ANOVA followed by Bonferroni's post test (Prism GraphPad, Inc.; San Diego, CA, USA) and significant differences were considered at $p < 0.05$.

Western blot protein expression was quantified on three different western blots using the ImageJ software version 1.45s and the means analyzed by one-way ANOVA and Bonferroni's post test as described above.

3 Results

3.1 Superoxide anion production induced by thiopurine drugs

To investigate the role of oxidative stress, the effects of AZA, 6-MP and 6-TG on ROS production, as superoxide anion, were evaluated in IHH and HCEC cells by the NBT reduction assay. As shown in figure 1, a significant superoxide anion production was detected only after AZA exposure in both cell lines, both after 8 and 24 h, exposure times after which no cytotoxic effect was observed. In particular, AZA induced a concentration-dependent superoxide anion increase starting from the concentration of 1.25×10^{-4} M. At the highest concentration of AZA (8.0×10^{-3} M), a 24 h treatment induced a release of superoxide anion equal to $82.7 \pm 6.8\%$ and $64.4 \pm 5.7\%$, in IHH (Panel A) and HCEC cells (Panel B), respectively. On the contrary, 6-MP and 6-TG did not induce significant effects on superoxide anion production.

3.2 Effect of allopurinol on thiopurines cytotoxicity

As shown in figure 2, thiopurines induced concentration-dependent cytotoxic effects after 96 h with EC_{50} values of 4.4×10^{-5} M (95% confidence intervals, $CI=2.8-7.0 \times 10^{-5}$ M), 1.1×10^{-4} M (95% $CI=0.8-1.5 \times 10^{-4}$ M) and 1.2×10^{-6} M (95% $CI=1.0-1.5 \times 10^{-6}$ M) for AZA, 6-MP and 6-TG, respectively, in IHH cells (Panel A). Similar results were obtained in HCEC cells (Panel B), in which 96 h treatment with thiopurines induced a concentration-dependent cytotoxic effect, with EC_{50} values of 7.4×10^{-5} M (95% $CI=5.8-9.4 \times 10^{-5}$ M), 1.8×10^{-4} M (95% $CI=1.2-2.5 \times 10^{-4}$ M) and 1.9×10^{-6} M (95% $CI=1.6-2.4 \times 10^{-6}$ M) for AZA, 6-MP and 6-TG, respectively. To evaluate the role of oxidative stress in thiopurines cytotoxicity, AZA, 6-MP and 6-TG were used in co-treatment with allopurinol, a well-known inhibitor of XO. IHH and HCEC cells

were pre-treated for 1 h with allopurinol 100 μ M and then exposed for 96 h to thiopurines. However, allopurinol co-exposure did not affect thiopurines cytotoxic effects.

3.3 Effect of allopurinol on thiopurines anti-proliferative effects

As shown in figure 3, only AZA and 6-TG induced a concentration dependent reduction of cell proliferation after 96 h exposure. In particular, in IHH cells (Panel A), cell proliferation was reduced with EC_{50} values of 1.1×10^{-5} M (95% CI= 0.8 - 1.7×10^{-5} M) and 2.6×10^{-6} M (95% CI= 2.3 - 3.1×10^{-6} M) for AZA and 6-TG, respectively. Similarly, in HCEC cells (Panel B), cell proliferation was reduced with EC_{50} values of 7.7×10^{-6} M (95% CI= 6.5 - 9.0×10^{-6} M) and 6.8×10^{-6} M (95% CI= 4.0 - 11.3×10^{-6} M) for AZA and 6-TG, respectively. To investigate the role of oxidative stress on thiopurines' anti-proliferative effects, IHH and HCEC cells were pre-exposed for 1 h to allopurinol 100 μ M and then exposed for 96 h to thiopurines. However, allopurinol co-treatment was able to reduce the anti-proliferative effect only of AZA in IHH cells, significantly increasing the EC_{50} value, equal to 7.1×10^{-5} M (95% CI= 4.6 - 10.9×10^{-5} M), by almost 7 times with respect to AZA alone ($p < 0.001$). Considering 6-MP, in both cell lines a concentration-dependent effect was not obtained and therefore it was not possible to calculate an EC_{50} value. However, allopurinol co-treatment was able to restore a concentration-dependent anti-proliferative effect induced by 6-MP only in IHH cells. Considering 6-TG, no inhibitory effect was observed in presence of allopurinol in both IHH ($EC_{50} = 3.6 \times 10^{-6}$ M; 95% CI= 2.9 - 4.7×10^{-6} M) and HCEC cells ($EC_{50} = 6.2 \times 10^{-6}$ M; 95% CI= 3.8 - 9.9×10^{-6} M).

3.4 Effect of N-acetyl cysteine on thiopurines cytotoxicity

To evaluate the role of GSH depletion in thiopurines cytotoxicity, AZA, 6-MP and 6-TG were used in co-treatment with N-acetyl cysteine (NAC), a synthetic precursor of GSH. IHH and HCEC cells were pre-treated for 1 h with NAC 1 mM and then exposed for 96 h to thiopurines. As shown in figure 4, NAC co-exposure did not affect thiopurines cytotoxic effects evaluated by MTT assay in both IHH (Panel A) and HCEC (Panel B) cells.

3.5 Effect of N-acetyl cysteine on thiopurines anti-proliferative effects

To investigate the role of GSH depletion on thiopurines' anti-proliferative effects, IHH and HCEC cells were pre-exposed for 1 h to NAC 1 mM and then exposed for 96 h to thiopurines. As shown in figure 5, only the antiproliferative effect of AZA, evaluated by ³H-thymidine incorporation assay, was specifically reduced in presence of NAC. In particular, in IHH cells (Panel A), the co-exposure with NAC significantly ($p < 0.001$) increased the EC_{50} value, equal to 9.0×10^{-5} M (95% CI = 5.4 – 15.0×10^{-5} M), about 8 times with respect to AZA alone. Similarly, in HCEC cells (Panel B), the co-exposure with NAC significantly ($p < 0.001$) increased the EC_{50} value, equal to 8.8×10^{-5} M (95% CI = 5.6 – 13.6×10^{-5} M), about 11 times with respect to AZA alone. On the contrary, in both cell lines, the co-treatment with NAC did not show significant change in response to 6-TG in terms of an antiproliferative effect. Again, considering 6-MP, in both cell lines a concentration-dependent response was not obtained.

3.6 Effects of azathioprine on glutathione depletion

To further investigate the role of GSH in AZA's effects, GSH was fluorimetrically quantified. Figure 6 shows the amount of GSH (expressed as a percentage of total GSH with respect to the untreated controls) in IHH (Panel A) and HCEC (Panel B) cells survived after AZA

exposure. Cells were exposed for 96 h to concentrations equal to the EC_{50} and EC_{95} values, corresponding respectively to 4.4×10^{-5} and 5.0×10^{-4} M for IHH cells and 7.4×10^{-5} and 5.0×10^{-4} M for HCEC cells. In both cell lines, 96 h exposure to AZA at a concentration equal to the EC_{95} value significantly reduced the amount of GSH. As a negative control, 6-MP did not affect GSH content after 96 h exposure (data not shown).

3.7 Effects of GST-M1 overexpression on thiopurines sensitivity

Both cell lines have been stable transfected with plasmids encoding for GST-M1. Once stably selected, cells were collected and lysed in order to extract the respective protein lysates. The transfection efficiency of the stable expression (Figure 7) was assessed by western blotting analysis, using anti-DDK antibodies on cellular extracts (Panel A), and quantification by densitometry analysis (Panel B). Both IHH and HCEC cell lines were transfected successfully, in particular the amount of protein expression, in terms of percentage of β -actin signal, was equal to $41.0 \pm 3.9\%$ and $59.2 \pm 4.3\%$ in IHH and HCEC cells, respectively.

The effect of GST-M1 overexpression on thiopurines-induced ROS production, as superoxide anion, after 24 h of treatment was evaluated in IHH and HCEC stably transfected cells. As shown in figure 8, in IHH cells (Panel A) AZA induced a concentration-dependent superoxide anion increase starting from the concentration of 8.0×10^{-6} M and, at the highest concentration of AZA (8.0×10^{-3} M), the treatment induced a release of superoxide anion equal to $78.7 \pm 8.3\%$, almost two times higher ($p < 0.001$) compared to that observed in cells transfected with the empty vector ($46.1 \pm 2.5\%$). In HCEC cells (Panel B) AZA induced a concentration-dependent superoxide anion increase starting from the concentration of 5.0×10^{-4} M and, at the highest concentration of AZA (8.0×10^{-3} M), the treatment induced a release of superoxide anion equal

to $96.4 \pm 5.3\%$, almost two times higher ($p < 0.001$) compared to that observed in cells transfected with the empty vector ($54\% \pm 0.9\%$).

Subsequently, the effect of GST-M1 over-expression on IHH and HCEC cells sensitivity to thiopurines has been assessed (figure 9). In IHH cells (Panel A) no significant difference was observed between the GST-M1 over-expressing and the empty-vector lines. On the contrary, in HCEC cells (Panel B) the forced expression of GST-M1 increased the sensitivity specifically to AZA. In particular, the EC_{50} value calculated in the GST-M1 transfected cells, equal to 4.2×10^{-5} M (95% CI = $2.9 - 6.0 \times 10^{-5}$ M), was about 5 times lower ($p < 0.001$) with respect to cells transfected with the empty vector ($EC_{50} = 1.9 \times 10^{-4}$ M; 95% CI = $1.4 - 2.8 \times 10^{-4}$ M). For 6-MP and 6-TG no significant differences were observed.

4 Discussion and Conclusions

This study was carried out with the aim to explain, from a mechanistic point of view, the clinical evidence that our group recently highlighted in young IBD patients demonstrating, for the first time, relevant effects of GST-M1 genotype on AZA effects. Indeed, GST-M1 deletion was associated with lower incidence of adverse events during AZA treatment,¹⁰ lower TGN/dose ratio and higher AZA dose requirement.¹¹ To this aim, the human non-tumor IHH and HCEC cell lines were chosen as predictive cell models of the hepatic and intestinal tissues, respectively, in which drug activation and some severe adverse effects of thiopurines occur.

Given the enzymes involved in thiopurines metabolism (GST, XO), it is reasonable to hypothesize that these drugs are able to induce oxidative stress conditions at the cellular level. This seems to be proven especially for AZA due to the GST-mediated depletion of GSH induced by its conversion to 6-MP,^{12, 17} possibly allowing the accumulation of significant amounts of ROS.^{15, 16} To oxidative stress, even 6-MP biotransformation by XO, a well-known ROS producer enzyme, as assessed on primary cultures of rat hepatocytes,¹⁸ could contribute.

In our model, AZA, but not 6-MP and 6-TG, induced a concentration-dependent ROS production, measured as superoxide anion, in the hepatic IHH and intestinal HCEC cell lines, after exposure times (i.e. 8 and 24 h) not inducing significant cytotoxic effects. This ROS increase was significative particularly at the highest concentrations of AZA, within the millimolar range. However, given the doses of thiopurines administered to patients and estimated volumes of intestinal and hepatic tissues, where they are well absorbed and undergo important biotransformations,⁴ it is likely that in these tissues thiopurines concentrations reach even the millimolar range.²² Intact AZA is undetectable in plasma after oral administration because of this extensive first pass metabolism, while for 6-MP, peak concentrations reach the

micromolar range.²³ The ability of AZA to induce oxidative stress is in line with previous studies demonstrating that thiopurines can generate directly ROS in cells exposed to biological doses of ultra violet light.^{24, 25} To understand the ROS contribution on the toxic effects of thiopurines, in particular of AZA, the effects of allopurinol, a selective inhibitor of XO, on thiopurines' cytotoxic and anti-proliferative effects were initially evaluated. Allopurinol has been shown to restore thiopurines response in IBD patients by increasing the concentration of active TGNs and decreasing that of the methylated nucleotides.²⁶ However, a relevant role for XO in mediating AZA-induced ROS production seems unlikely. Indeed, AZA cytotoxic effects were not reduced in presence of allopurinol at a concentration inhibiting XO activity.²⁷ An effect was observed on the anti-proliferative action induced only on IHH cells, probably because of a higher expression of XO with respect to the intestinal cell line. In particular, allopurinol co-treatment significantly increased the EC₅₀ value by almost 7 times with respect to AZA alone and, interestingly, was able to restore the concentration-dependency of the effect induced by 6-MP. Indeed, in both cell lines, 6-MP was unable to induce a proper concentration-dependent anti-proliferative effect since at the highest concentrations (10^{-3} – 10^{-2} M), a proliferation increase was observed. Even if a complete study to explain this aberrant response was not carried out, we can hypothesize that the reasons could reside in some effects on the regulation of cell cycle. As previously demonstrated in other cell models, 6-MP concentrations higher than 0.5×10^{-3} M seem able to modulate a drug-induced block of cell cycle that could tentatively result in hindering 6-MP *in vitro* effects.²⁸ In accordance with the fact that 6-TG is not a direct substrate for XO,²⁹ no difference in the EC₅₀ were observed for 6-TG in presence of allopurinol.

Since the contribution of XO seems only partial, our attention was focused on the role of GSH depletion involved in the metabolic conversion of AZA into 6-MP. To this aim, the effects of the GSH precursor NAC co-exposure were studied. Our results demonstrated that NAC co-

exposure was able to reduce the anti-proliferative, but not the cytotoxic effects, specifically of AZA. On the contrary, no significant effects of NAC co-treatment were found for 6-MP and 6-TG in both cell lines. This result is in line with the ability of AZA, but not of the other thiopurines, to reduce the amount of GSH and to induce a concentration-dependent ROS production in both cell lines. On the whole, these results are in good agreement with a very recent study carried out on primary cultures of rat hepatocytes.³⁰ In our model, the contribution of oxidative stress appears to be greater on the anti-proliferative effects of AZA compared to cytotoxicity. This is probably due to the ability of ROS to modulate cell cycle.^{31, 32} Alternatively, the anti-proliferative effect may be a sign of an early cellular toxicity and therefore still reversible by NAC co-treatment.

Finally, to corroborate the role of GST-mediated GSH depletion in AZA mechanism of action, a stable over-expression of GST-M1 was carried out. Our focus was directed on M1 isoform because of the clinical evidence that GST-M1 deletion is associated to a reduced sensitivity to therapy in IBD patients.^{10, 11} In our model, the increased expression of GST-M1 increased AZA cytotoxicity in HCEC cells, while no effects were observed for 6-MP and 6-TG, in line with the fact that GST enzymes seem to have a significant role on AZA's, but not other thiopurine's, toxicity. This effect could be due to the increased rate of conversion of AZA to 6-MP in HCEC cells over-expressing GST-M1, which, hypothetically, leads to increased concentrations of the active thioguanine metabolites. Interestingly, in the GST-M1 over-expressing cell line an increased superoxide anion production was observed with respect to the native cell line, corroborating the role of ROS due to the greater activation of AZA by GST-M1. In the IHH cell line, however, the increased expression of GST-M1 does not determine a greater cytotoxicity for any of the thiopurines, although an increase of oxidative stress induced by AZA was observed. The lack of effects of the GST-M1 over-expression on the cytotoxicity of AZA in transfected IHH cells could be due to the high levels of this

enzyme already in the native line, while it is absent, both in terms of mRNA and protein, in the HCEC cell line (data not shown). The native level of GST-M1 could be sufficient to determine the rapid conversion of AZA into 6-MP, and consequently, the increase in enzyme levels induced by the forced expression of GST-M1 could be irrelevant. Notwithstanding, in this study an *in vitro* model has been set up to study AZA metabolism and could be further supported by silencing of GST-M1 activity. However, this model seems to be more informative for AZA with respect to the other thiopurines, given the more prominent role of GSH in AZA toxic effects. This *in vitro* model helped us to demonstrate, for the first time, a clear role of GST-M1 in modulating AZA cytotoxicity with a close dependency on ROS production. On the contrary, this study demonstrates that GST-M1 doesn't have a significant role in modulating the other thiopurines (6-MP and 6-TG) cytotoxicity, for which ROS production doesn't seem to be involved in their mechanism of cytotoxicity.

In conclusion, in this study it has been demonstrated the contribution of oxidative stress to the anti-proliferative effects of AZA in the human IHH and HCEC cell lines, derived respectively from the liver and intestinal tissues. This ROS production seems to be mediated by GSH depletion occurring during the conversion of AZA to 6-MP catalyzed by GST enzymes and, only in part, by XO enzymes during 6-MP metabolism. A prominent role of GST, isoform M1, was clearly demonstrated in stably increased GST-M1 expression, especially in the intestinal HCEC cell line, lacking this enzyme in its native form, where GST-M1 over-expression increased AZA cytotoxicity as well as ROS production (figure 10). Even if the amount of active metabolites should be evaluated to investigate if the increased sensitivity is due also to an enhanced metabolism, besides to an enhanced ROS production, this study provides the molecular basis to explain the clinical evidence suggesting a prominent role of GST-M1 genotype in influencing AZA clinical effects.

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Notes

The authors declare no competing financial interest.

Abbreviations

6-MP (6-mercaptopurine), 6-TG (6-thioguanine), 6-TGNs (thioguanine nucleotides), AZA (azathioprine), IBD (inflammatory bowel disease), GSH (glutathione), GST (glutathione-S-transferase), NAC (N-acetyl cysteine), ROS (reactive oxygen species), TPMT (thiopurine-S-methyltransferase), XO (xanthine oxidase)

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Figure legends

Fig. 1. Effect of AZA, 6-MP and 6-TG on superoxide anion production. IHH (Panel A) and HCEC cells (Panel B) were exposed for 8 and 24 h to scalar concentrations of AZA, 6-MP and 6-TG before performing the NBT assay. The data are reported as % increase of superoxide anion with respect to negative controls (untreated cells) and are the means \pm SE of 5 independent experiments performed in triplicate. Statistical difference: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (treated vs untreated cells, one-way ANOVA and Bonferroni's post test).

Fig. 2. Effect of allopurinol on AZA, 6-MP and 6-TG cytotoxicity in IHH (Panel A) and HCEC (Panel B) cells. Cells were exposed for 1 h to allopurinol 100 μ M followed by 96 h to thiopurines and cytotoxicity evaluated by MTT assay. The data are reported as means \pm SE of 4 independent experiments performed in triplicate.

Fig. 3. Effect of allopurinol on AZA, 6-MP and 6-TG anti-proliferative effects in IHH (Panel A) and HCEC (Panel B) cells. Cells were exposed for 1 h to allopurinol 100 μ M followed by 96 h to thiopurines and cell proliferation evaluated by the 3H-thymidine incorporation assay. The data are reported as means \pm SE of 4 independent experiments performed in triplicate. Statistical difference: **, $p < 0.01$; ***, $p < 0.001$ (with vs without allopurinol, two-way ANOVA and Bonferroni's post test).

Fig. 4. Effect of N-acetyl-cysteine (NAC) on AZA, 6-MP and 6-TG cytotoxicity in IHH (Panel A) and HCEC (Panel B) cells. Cells were exposed for 1 h to NAC 1 mM followed by 96 h to thiopurines and cytotoxicity evaluated by MTT assay. The data are reported as means \pm SE of 4 independent experiments performed in triplicate.

Fig. 5. Effect of N-acetyl-cysteine (NAC) on AZA, 6-MP and 6-TG anti-proliferative effect in the IHH (Panel A) and HCEC (Panel B) cells. Cells were exposed for 1 h to NAC 1 mM followed by 96 h to thiopurines and cell proliferation evaluated by the 3H-thymidine

incorporation assay. The data are reported as means \pm SE of 4 independent experiments performed in triplicate. Statistical difference: **, $p < 0.01$; ***, $p < 0.001$ (with vs without NAC, two-way ANOVA and Bonferroni's post test).

Fig. 6. Effect of AZA on GSH depletion. IHH (Panel A) and HCEC (Panel B) cells were exposed for 96 h to AZA and the amount of GSH measured fluorimetrically in the survived cells. Data are presented as percentage of total GSH with respect to the untreated controls and are the mean \pm SE of 3 experiments performed in duplicate. Statistical difference: **, $p < 0.01$ (treated vs untreated cells, two-way ANOVA and Bonferroni's post test).

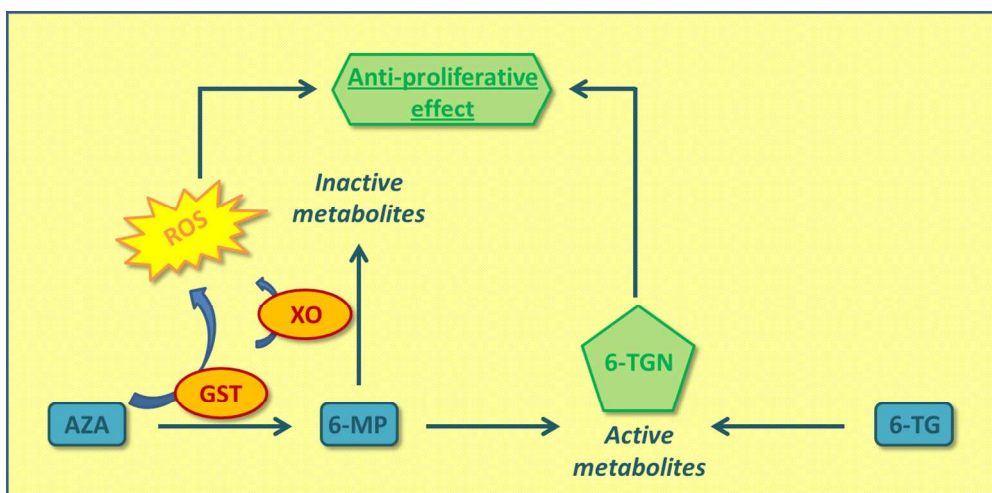
Fig. 7. IHH and HCEC cell lines have been stable transfected with plasmids encoding for GST-M1. Western blot using anti-DDK antibodies on cellular extract (Panel A) and quantification by densitometry analysis (Panel B) has been assessed. The results were compared to those obtained in cells transfected with the empty vector (MOCK) and DDK expression is reported as a percentage with respect to β -actin. The data are reported as means \pm SE of 3 independent experiments.

Fig. 8. Effect of GST-M1 overexpression on superoxide anion production induced by AZA, 6-MP and 6-TG in IHH (Panel A) and HCEC (Panel B) stably transfected cells. Cells were exposed for 24 h to scalar concentrations of AZA, 6-MP and 6-TG before performing the NBT assay. The results were compared to those obtained in cells transfected with the empty vector (MOCK). The data are reported as % increase of superoxide anion with respect to negative controls (untreated cells) and are the means \pm SE of 4 independent experiments performed in triplicate. Statistical difference: *, $p < 0.05$; ***, $p < 0.001$ (GST-M1 vs MOCK, two-way ANOVA and Bonferroni's post test).

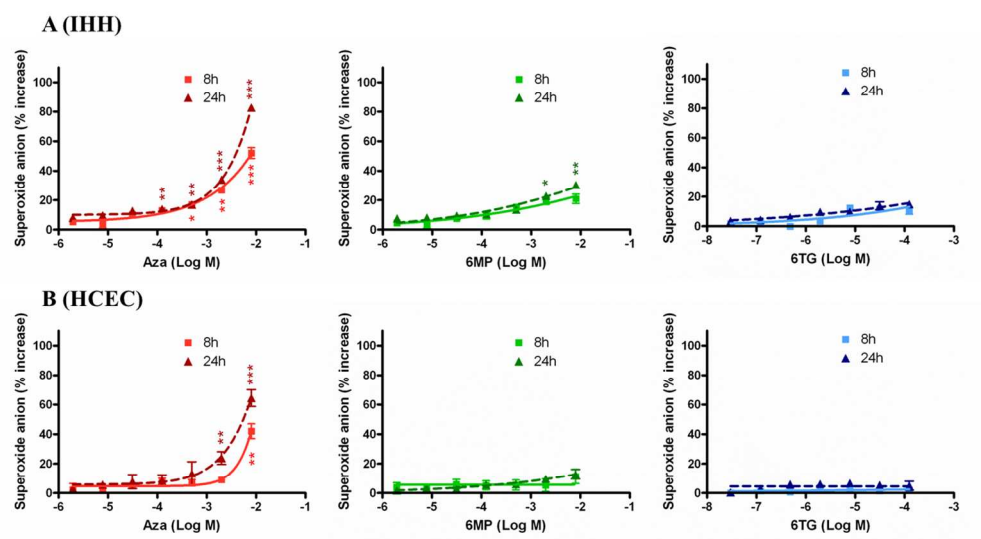
Fig. 9. Effect of GST-M1 overexpression on AZA, 6-MP and 6-TG cytotoxicity in IHH (Panel A) and HCEC (Panel B) stably transfected cells. Cells were exposed for 96 h to scalar

concentrations of AZA, 6-MP and 6-TG and cytotoxicity evaluated by MTT assay. The results were compared to those obtained in cells transfected with the empty vector (MOCK). The data are reported as means \pm SE of 4 independent experiments performed in triplicate. Statistical difference: **, $p < 0.01$ (GST-M1 vs MOCK, two-way ANOVA and Bonferroni's post test).

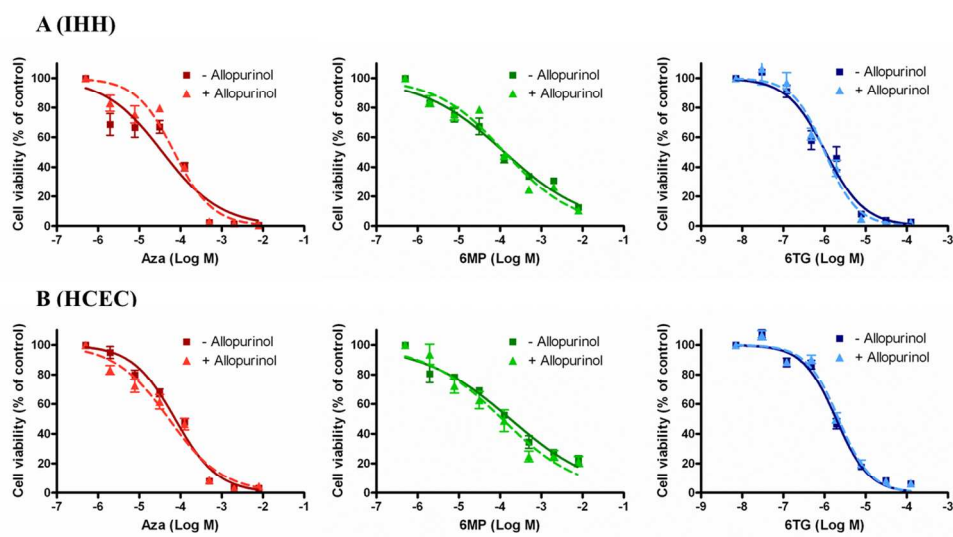
Fig. 10. Proposed mechanism of ROS production induced by thiopurines metabolism. ROS production seems to be mediated by GSH depletion occurring during the conversion of AZA to 6-MP catalyzed by GST enzymes and, only in part, by XO enzymes during 6-MP metabolism. In our study we focused on GST-M1 isoform. (6-MMP=6-methyl-mercaptopurine; 6-MP=6-mercaptopurine; 6-TG=6-thioguanine; 6-TGDP=6-thioguanine-diphosphate; 6-TGMP=6-thioguanine-monophosphate; 6-TGN=6-thioguanine nucleotide; 6-TGTP=6-thioguanine-triphosphate; 6-TU=6-thiouric; AZA=azathioprine; GST-M1=glutathione-S-transferase M1; K=kinase; TPMT=thiopurine-S-methyl-transferase; XO=xanthine-oxidase)



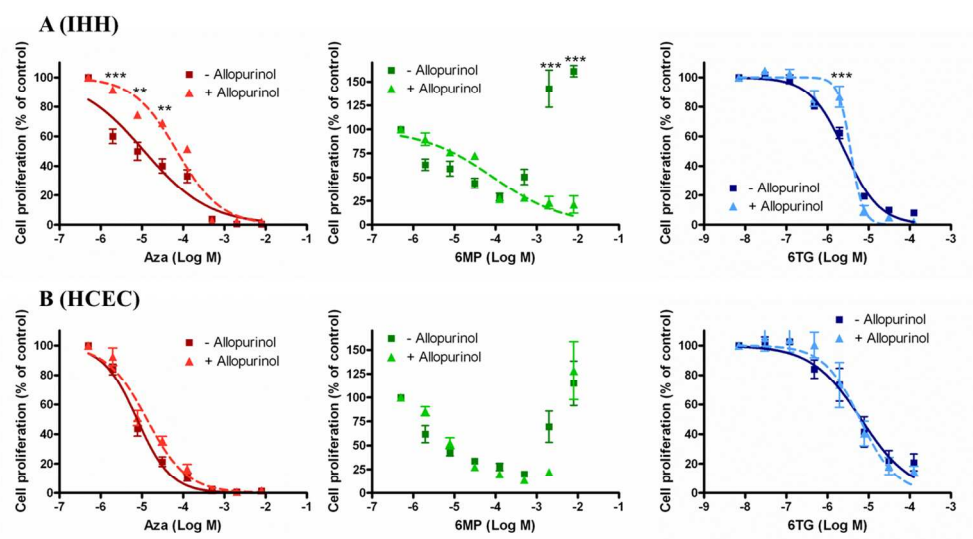
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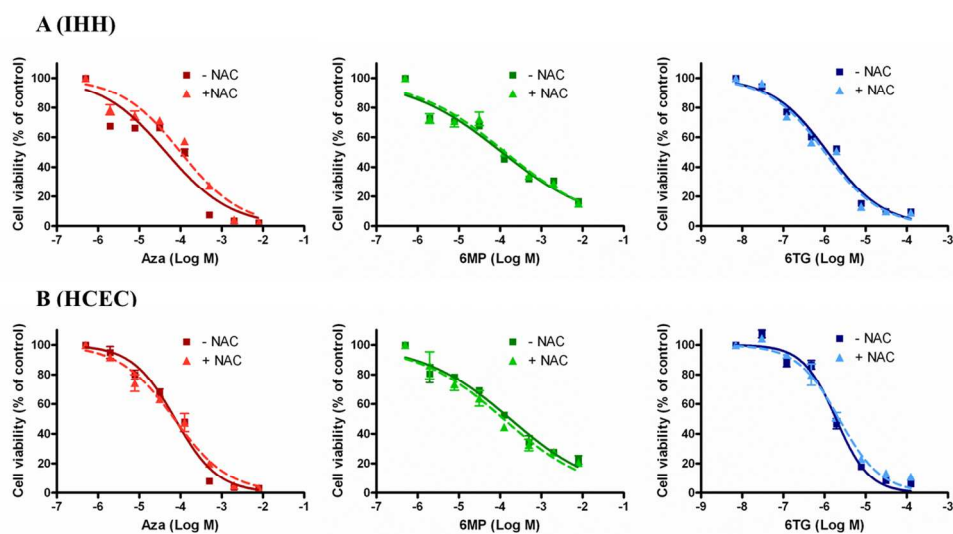
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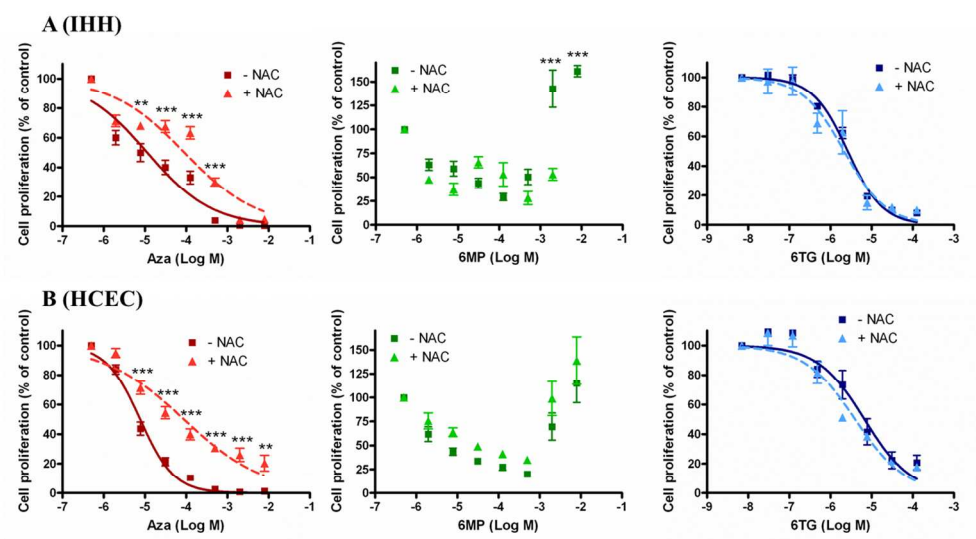
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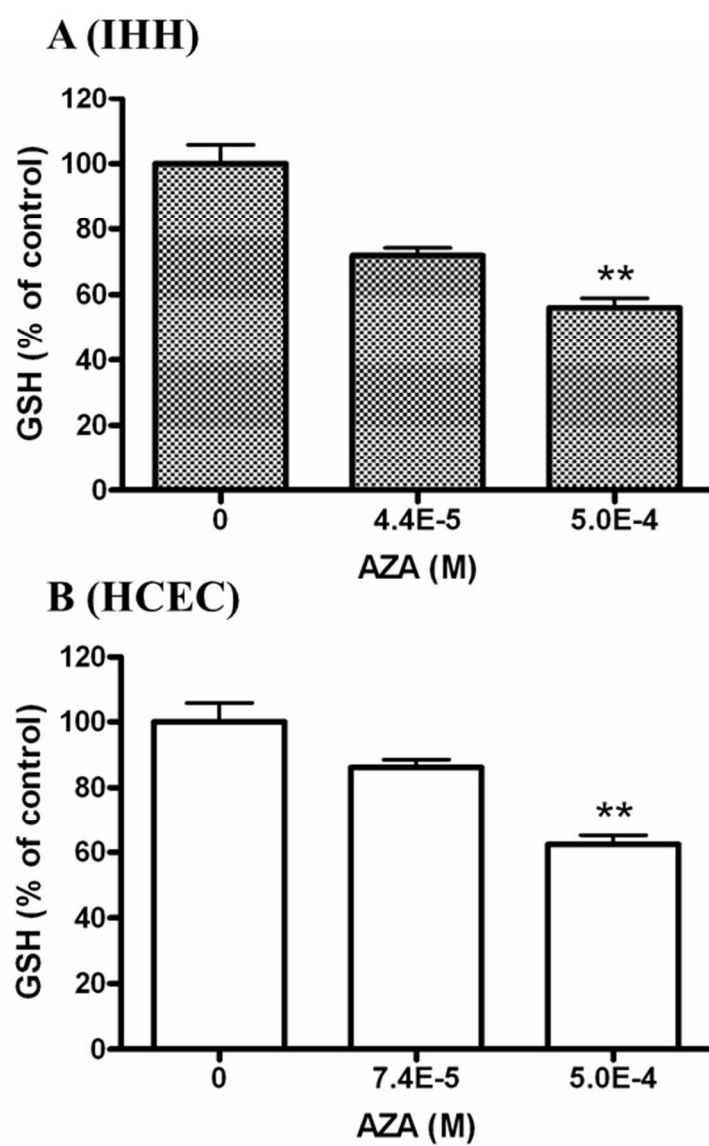
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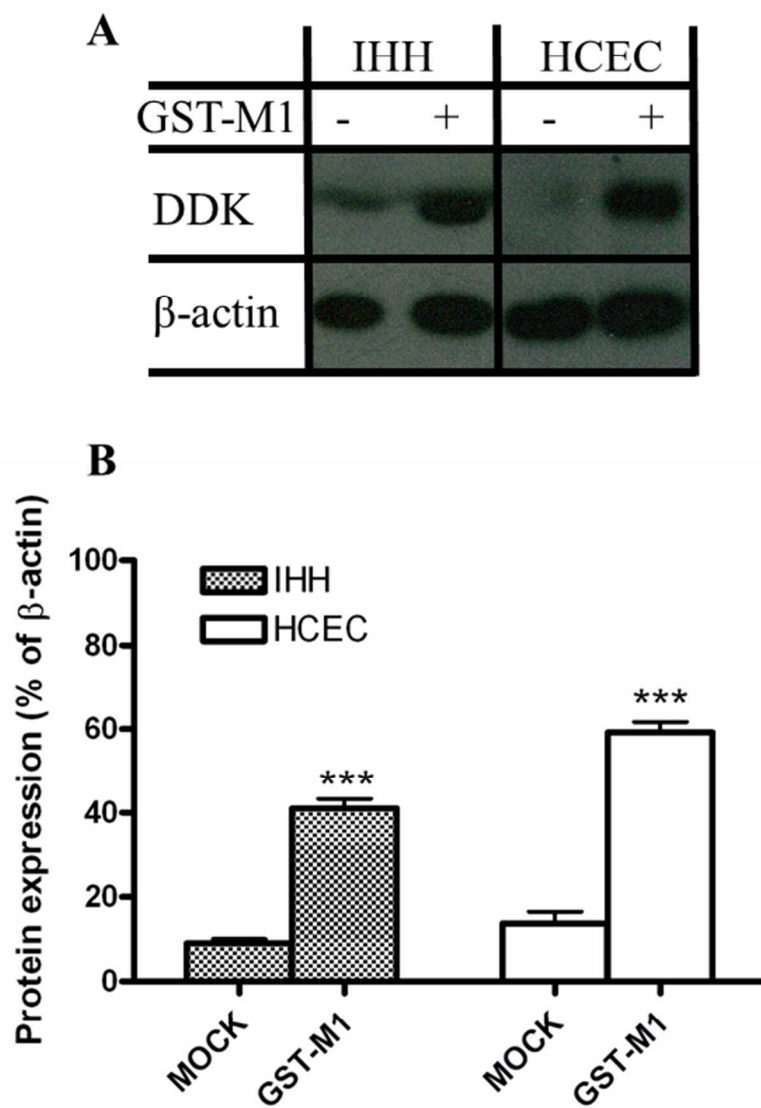
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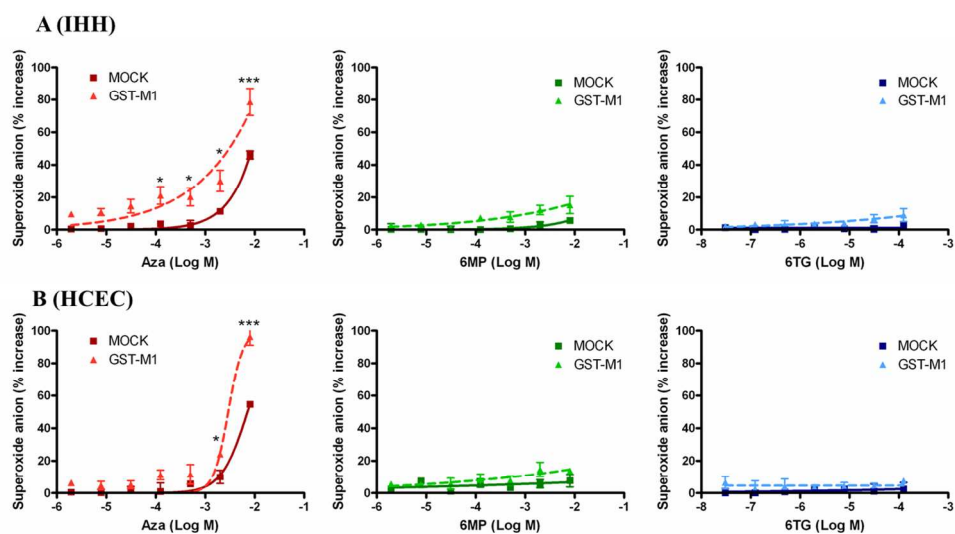
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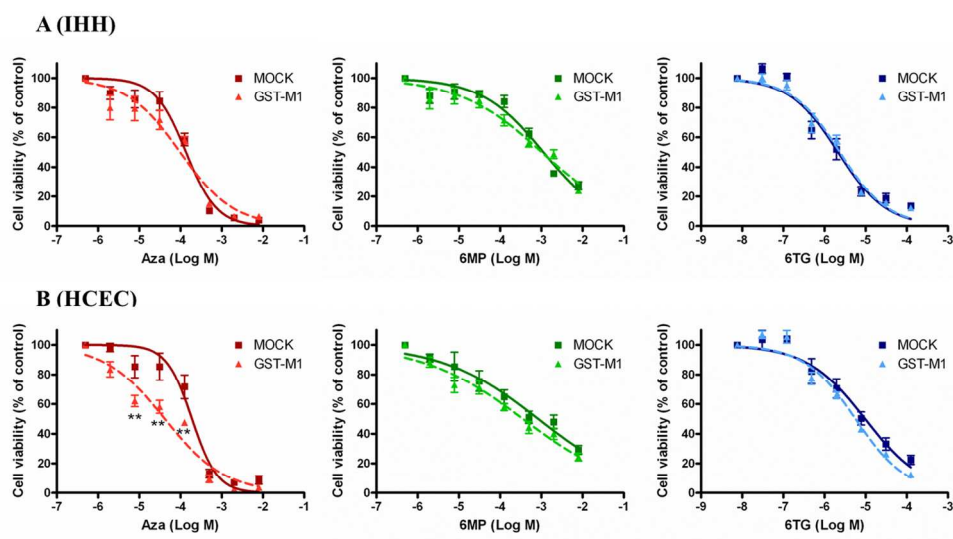
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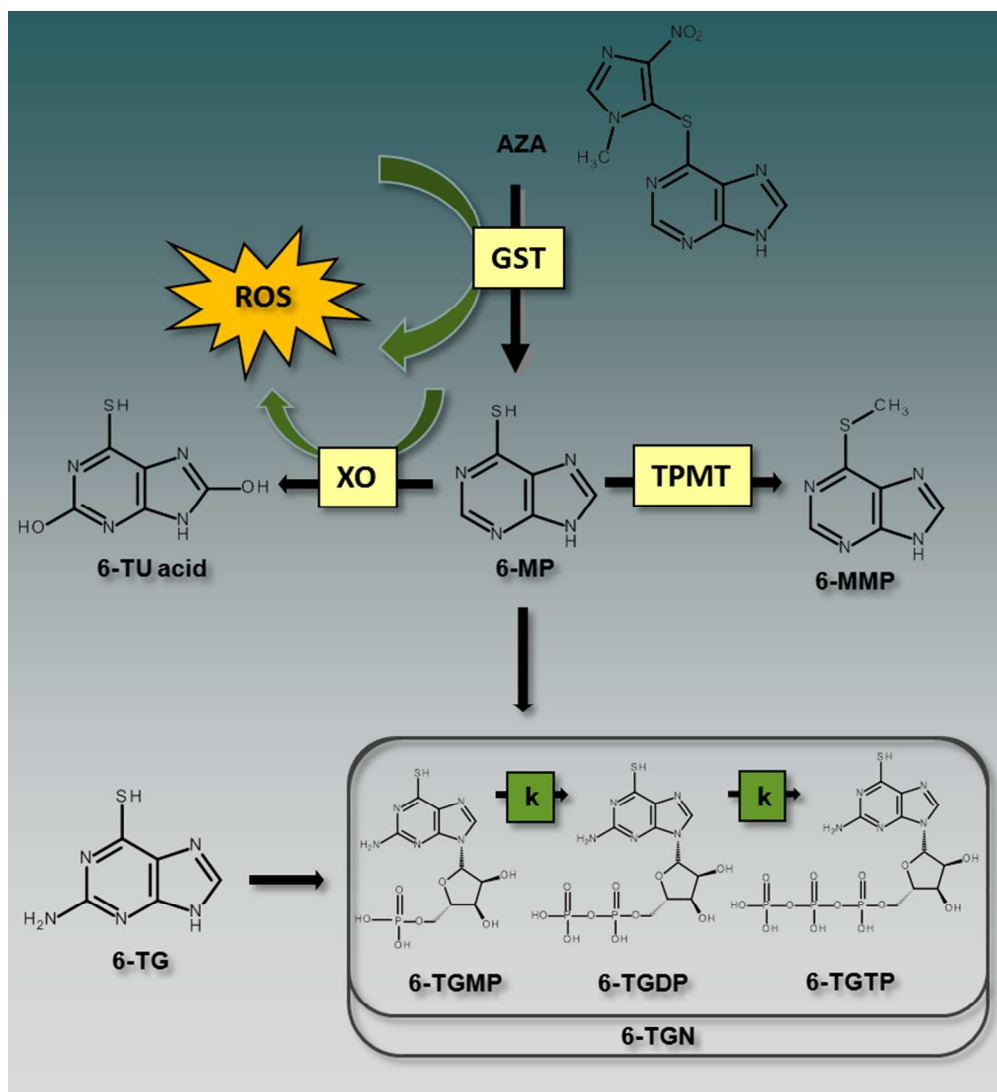
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